FLUORESCENT LABELS FOR ANTIBODY PROTEINS. APPLICATION TO BACTERIAL IDENTIFICATION[1]

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The early work on the use of fluorescent labeled antisera in immunology was performed by Coons *et al.* (1942) and Coons and Kaplan (1950). For a review up to 1954 see Coons (1954). This defined technique permits selective "staining" of antigens either in tissue section or isolated on a glass slide.

Of the different compounds available for labeling purposes, fluorescein (as the isocyanate derivative) has been most widely used. However, this compound, as well as rhodamine B isocyanate (Silverstein, 1957), requires specialized chemical equipment for its preparation and is not available commercially, probably because of its relative instability. Weber (1952), in an investigation of polarization of the fluorescence of macromolecules, described the labeling of proteins with 5-dimethylamino-1-naphthalene sulfonyl chloride (DNS).[2] This compound, available commercially (California Foundation for Biochemical Research, 3408 Fowler St., Los Angeles 63, California), is stable and requires no chemical treatment prior to use. Clayton (1954) in a brief note described the use of what is probably this compound in the localization of embryonic antigens, and Hartley and Massey (1956) have used it in studies on the active center of chymotrypsin. Not every laboratory has the facilities for the ultraviolet light illumination required ordinarily for work with labeled fluorescent antibodies. Therefore, the use of equipment which may already be at hand and which requires only minor modification would be desirable. As shown below, a commercially available microprojector (Scopicon, manufactured by Scopicon, Inc., New York, N. Y.), used mainly for clinical-pathological teaching and conference work, can be adapted readily for illuminating preparations to be observed for fluorescence. DNS, among a number of fluorescent compounds, was the only replacement found for fluorescein.

MATERIALS AND METHODS

The indirect method of labeling used (Weller and Coons, 1954) requires antiserum to rabbit globulin. Rabbit serum globulin was prepared as follows. The crude globulin fraction of rabbit serum was precipitated at 5 C with ammonium sulfate at 50 per cent saturation. This fraction was dissolved in a measured volume of water and reprecipitated by an equal volume of saturated ammonium sulfate solution. This cycle was repeated twice more. The globulin was then dialyzed against normal saline (2 L per 100 ml globulin solution) for 3 days, the dialyzing fluid being changed twice daily. The dialyzed globulin was stored at −20 C.

A rabbit globulin adjuvant mixture was prepared by adding 10 ml of rabbit globulin solution (of about twice normal blood concentration) in small increments to 10 ml of adjuvant (Freund).[3] After each addition, the mixture was repeatedly aspirated into, and expelled from, a 10-ml syringe. In this way, a creamy white emulsion was produced which gradually separated on standing but which was readily redispersed on mild shaking.

Three female goats were each injected into the

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[2] While this manuscript was in preparation, a paper by Redetzki (1958), on the labeling of antibodies by DNS, appeared.

[3] Kindly supplied by Mr. A. L. Lane, Difco Laboratories, Detroit, Michigan. Control no. 90093.

thigh with 3 ml of adjuvant-globulin mixture. These injections were repeated 14 days later, and bleedings were made 12 to 19 days thereafter from an external jugular vein. Three bleedings were made over 1 week.

The separated goat serum was then fractionated for its globulin content as described for rabbit globulin and this fraction constituted the anti-rabbit globulin. Up to 5 cycles of solution and reprecipitation were carried out; dialysis was prolonged to 5 days and the volume of dialysis fluid was 8 L per change. Normal goat serum was fractionated in a similar manner.

Protein was determined by a biuret method using bovine serum albumin (Armour), fraction V, crystalline, as a standard.

The optical equipment included a cardioid condenser used in conjunction with an apochromatic oil immersion objective (with variable iris) and a 10× eyepiece to which (or in which) was attached a barrier filter (e. g., Leitz 2.5 mm) to screen out the illuminating light.

Light from an A-H 6 (General Electric Company) water cooled, high pressure mercury lamp, suitably housed and situated below the microscope, was projected by means of a simple lens (53 mm diameter, 50 mm focal length) through the filter system ($CuSO_4$ solution, followed by Wratten no. 5840, ½ standard thickness, or no. 9863) into the microscope. However, the Scopicon microprojector, which used the A-H 6 lamp, was found to be much more satisfactory because of its ease of adjustment. The self-contained water cooling unit is an added convenience. The only addition required was a water-cooled liquid filter, to hold $CuSO_4$ solution for removal of the red portion of the spectrum, as well as for minimizing the chances for thermal cracking of the ultraviolet isolating filters. The water lines to and from the Hg lamp were fitted with T connections for supplying cooling water to this filter cell. The concentration of the $CuSO_4$ was adjusted to the point where no red light was apparent in the light beam after passage through the ultraviolet filter.

Fluorescein amine, a gift from Dr. Coons, was converted to the isocyanate and coupled to anti-rabbit globulin as described by Coons and Kaplan (1950). Other compounds[4] were added in small quantities, in acetone solution, to cooled protein solutions and left standing overnight at 4 C. Any precipitate was removed by centrifugation and the excess labeling agent removed by dialysis against 0.2 N KCl until a negligible amount of fluorescing material was detectable in the dialyzate. Quantitative measurements of fluorescence were made in a Coleman Universal spectrophotometer with the equipment supplied for this operation.

Smears of bacterial cultures were made on glass slides, air dried, and fixed for 20 min in methanol. The indirect technique of labeling was carried out as follows: specific antiserum, rabbit origin, was placed on the dried smear and allowed to remain for ½ to 1 hr. In this and the succeeding step, preparations were held at room temperature in petri dishes containing a pad of water-soaked filter paper. Unreacted protein was then gently washed off with saline-buffer and the labeled anti-rabbit globulin added. This was rinsed off after ½ to 1 hr and the slide dried in air. The preparation was observed either in a glycerol-buffer mount under a cover slip, or directly under oil.

RESULTS

A variety of fluorescent aromatic sulfonic acids and their sulfonyl chloride derivatives,[4] other than DNS, at pH approximately 5 or 7.5, did not couple to protein or at a rate that was too low to measure. Some were retained by the cellulose dialysis membrane in the absence of protein.

Addition of graded amounts of DNS were made to 5 per cent Bovine gamma globulin (Armour). After removal of uncombined DNS, fluorophotometric measurements revealed that 0.1 to 0.6 per cent (w/v) DNS yielded essentially the same degree of labeling. Anti-rabbit globulin, treated similarly, was found to be reactive serologically when applied to smears of *Pasteurella pestis* strain A1122 using the indirect technique. This label, in contrast to fluorescein, rapidly bleached during observation with ultraviolet illumination. However, the rate of dis-

[4] 1-Amino-2-naphthol-4-sulfonic acid; 4-amino-2-naphthalenesulfonic acid; 1-naphthol-4-sulfonic acid; 3,4-diamino-2,7-naphthalenedisulfonic acid; 3-(4-anilino-1-naphthazo)-2,7-naphthalenedisulfonic acid (NH_3); Benzopurpurin 4B; Alizarine Red S; 2-anthraquinonesulfonic acid (Na); the foregoing were obtained from Eastman Kodak. DNS (original sample) was a gift from the California Foundation for Biochemical Research.

appearance was not so high that adequate observations could not be made.

Either the modified Scopicon projector or the laboratory-built light source was satisfactory for illuminating the specimens.

DISCUSSION

Although the fluorescent label DNS described has been tried with but one antigen-antibody system, this should not detract from more general application, since it is merely a replacement for the more commonly used fluorescein.

Bleaching of the DNS label under ultraviolet excitation should not be considered to be a disadvantage. Indeed, this very feature may be of value in deciding between specific and nonspecific fluorescence since nonspecific staining does not bleach.

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SUMMARY

A commercially available fluorescent compound, 5-dimethylamino-1-naphthalene sulfonyl chloride, which is relatively stable and does not require further chemical alteration was shown to be a replacement for fluorescein isocyanate as a labeling agent for specific antisera, when tested for identification of *Pasteurella pestis*. A commercially available light source, part of the Scopicon microprojector, may be easily adapted for use with fluorescent antibody techniques.

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